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Anaplasma phagocytophilum MSP2(P44)-18 Predominates and Is Modified into Multiple Isoforms in Human Myeloid Cells▽

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Anaplasma phagocytophilum **is the etiologic agent of human granulocytic anaplasmosis. MSP2(P44), the bacterium's major surface protein, is encoded by a paralogous gene family and has been implicated in a variety of pathobiological processes, including antigenic variation, host adaptation, adhesion, porin activity, and structural integrity. The consensus among several studies performed at the DNA and RNA levels is that a heterogeneous mix of a limited number of *msp2*(*p44*) transcripts is expressed by *A. phagocytophilum* during in vitro cultivation. Such analyses have yet to be extended to the protein level. In this study, we used proteomic and molecular approaches to determine that MSP2(P44)-18 is the predominant if not the only paralog expressed and is modified into multiple 42- to 44-kDa isoforms by *A. phagocytophilum* strain HGE1 during infection of HL-60 cells. The *msp2*(*p44*) expression profile was homogeneous for *msp2*(*p44*)*-18*. Thus, MSP2 (P44)-18 may have a fitness advantage in HL-60 cell culture in the absence of selective immune pressure. Several novel 22- to 27-kDa MSP2 isoforms lacking most of the N-terminal conserved region were also identified. *A. phagocytophilum* MSP2(P44) orthologs expressed by other pathogens in the family *Anaplasmataceae* are glycosylated. Gas chromatography revealed that recombinant MSP2(P44)-18 is modified by glucose, galactose, xylose, mannose, and trace amounts of other glycosyl residues. These data are the first to confirm differential modification of any *A. phagocytophilum* MSP2(P44) paralog and the first to provide evidence for expression of truncated versions of such proteins.**

Anaplasma phagocytophilum is an obligate intracellular bacterium and the causative agent of human granulocytic anaplasmosis, an emerging infectious disease in the United States, Europe, and Asia. It is also a veterinary pathogen. The bacterium is naturally maintained in zoonoses between its animal reservoirs and *Ixodes* species tick vectors. Within its mammalian host, *A. phagocytophilum* exhibits an unusual tropism for granulocytes (10, 15). Clinical disease associated with human granulocytic anaplasmosis is usually acute, though long-term adverse health outcomes such as recurrent fevers even after antibiotic treatment have been reported (37). Manifestations associated with this potentially fatal infection include fever, headache, myalgia, anorexia, and chills. These are often accompanied by leukopenia, thrombocytopenia, and elevations in serum hepatic aminotransferases (10, 15).

A. phagocytophilum possesses a series of polymorphic genes known as the major surface protein 2 (*msp2*; *p44*) family that encode 42- to 49-kDa transmembrane proteins carrying immunodominant B-cell epitopes. MSP2(P44) proteins share a common structure consisting of a central hypervariable region (HVR) flanked by conserved N- and C-terminal regions. The HVRs exhibit strong predictability of being surface exposed, thus providing potential sources for surface phenotype diversity (20, 33, 49). The *A. phagocytophilum* MSP2(P44) proteins are orthologous to those encoded by the *Anaplasma marginale msp2* family (34). *A. marginale* remains within its ruminant host for the animal's lifetime, and MSP2 antigenic variation is directly linked to the bacterium's persistence in vivo (11, 34). Likewise, the *A. phagocytophilum* MSP2(P44) proteins are believed to afford antigenic variation (10, 15), and a recent study that followed the *msp2*(*p44*) transcriptional profile of a clonal *A. phagocytophilum* population over the course of equine infection supports this hypothesis (26). Diversity in MSP2(P44) results from RecF-mediated gene conversion of a single genomic *msp2*(*p44*) expression site by partially homologous sequences (1–3, 27, 28). The *A. phagocytophilum* genome contains a large reserve (113 in the sequenced strain, HZ) of *msp2*(*p44*) genes or gene fragments lacking 5′- or 3′-terminal sequences (18). The gene fragments have been referred to as "functional pseudogenes" (26) because, rather than being nonfunctional fragments on their way to elimination, they recombine into the *msp2*(*p44*) expression site to yield full-length *msp2*(*p44*) paralogs. This has been demonstrated by Southern blotting (27) and by sequencing PCR products generated using primers targeting the *msp2*(*p44*) expression site (2, 3, 26, 27) and reverse transcription-PCR (RT-PCR) products generated using HVR-specific primers (26, 27, 40, 45). Proteomic characterization of MSP2(P44) paralogs has not been performed.

Amino acid similarity among the HVRs ranges from 19.9 to 32.8% (47). Despite such variability, conservation of certain

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"signature" residues and conservative replacements with respect to charge or size within HVRs are proposed to maintain proper MSP2(P44) folding (2, 3, 29). This, combined with observations that certain *A. phagocytophilum* MSP2(P44) paralogs (3, 22, 23, 29, 47, 48) as well as specific orthologs of the P28 and P30 outer membrane protein families of other *Anaplasmataceae* pathogens, *Ehrlichia chaffeensis* and *Ehrlichia canis* (42, 43), respectively, are expressed predominantly within tick or mammalian environments has led to the speculation that these proteins may also play important roles in host adaptation, including adhesion to particular host cell types (36). Porin activity has also been attributed to MSP2(P44) (19). Individual *E. chaffeensis* P28 and *E. canis* P30 proteins are glycosylated and phosphorylated into multiple expressed forms (42, 43). Several additional *Anaplasmataceae* outer membrane proteins (*A. marginale* MSP1a [13] and *E. canis* gp19, gp36, and P140 and *E. chaffeensis* gp47 and P120 [9, 30, 31]) are also glycosylated. Adherence of recombinant MSP1a to tick cells has been attributed to its glycosylation (13). It is unclear whether *A. phagocytophilum* MSP2(P44) is also posttranlsationally modified.

In this study, we assessed *A. phagocytophilum* MSP2(P44) expression and posttranslational modification in human myeloid cell culture. MSP2(P44)-18 is the predominant if not the only paralog expressed and is modified into multiple 42- to 44-kDa isoforms during *A. phagocytophilum* strain HGE1 development in HL-60 cells. Notably, several 22- to 27-kDa isoforms lacking a majority of the N-terminal conserved region of MSP2(P44)-18 were also identified. As has been determined for recombinant forms of other *Anaplasmataceae* pathogen surface proteins (13, 31), glycosyl residues, the primary of which are glucose, galactose, mannose, and xylose, decorate recombinant MSP2(P44)-18 [rMSP2(P44)-18]. These data provide the first evidence for the expression of multiple MSP2 (P44) isoforms, MSP2(P44) glycosylation, and expression of truncated MSP2(P25) proteins in *A. phagocytophilum*.

MATERIALS AND METHODS

In vitro cultivation of *A. phagocytophilum*. *A. phagocytophilum* HGE1 (a gift from Ulrike Munderloh and Michael Herron of the University of Minnesota) was cultured in HL-60 cells (5). The HGE1 culture had been passed 92 times in HL-60 cells at the initiation of this study.

Preparation of *A. phagocytophilum* outer membrane fractions. Host cell-free bacteria were prepared as described previously (5). Bacterial outer membrane fractions were enriched for by temperature-dependent Triton X-114 phase partitioning (4, 39) using the Membrane I ReadyPrep protein extraction kit (Bio-Rad, Hercules, CA) with minor modifications to the manufacturer's protocol. Five hundred microliters of ice-cold buffer M1 supplemented with complete protease inhibitors (Roche Applied Science, Indianapolis, IN) was used to resuspend a 50-μl wet *A. phagocytophilum* cell pellet. The suspensions were subjected to five 30-second bursts on ice using a Misonix S3000 ultrasonic processor at a power setting of 2. Suspensions were chilled on ice for 1 min between sonication treatments. An equal volume of ice-cold buffer M2 was added, and samples were vortexed 5 times for 60 seconds interspersed with 10 min incubations in an ice-water bath. Samples were incubated in a 37°C water bath for 30 min, during which they were vortexed every 7.5 min. Centrifugation at 16,000 × *g* for 5 min at 25°C partitioned the sample into an upper hydrophilic phase, a lower hydrophobic phase, and a hydrophobic pellet. The hydrophilic phase was transferred to a new tube. Five hundred microliters of ice-cold buffer M2 was added to the hydrophobic protein fraction, followed by a second round of hydrophilic protein extraction. The two hydrophilic fractions were combined and stored at −80°C. The hydrophobic liquid and hydrophobic pellet fractions were individually stored at −80°C. Protein concentrations in each fraction were determined using the RC-DC protein assay (Bio-Rad).

Two-dimensional SDS-polyacrylamide gel electrophoresis (2DE) and gel staining. Detergents and other impurities were removed from *A. phagocytophilum* hydrophobic pellet or liquid fractions using the ReadyPrep 2-D cleanup kit (Bio-Rad). Thirty-five micrograms of protein sample was resolved at 10°C by isoelectric focusing (IEF) in a Mulitphor II electrophoresis system (Amersham Pharmacia Biotech, Piscataway, NJ) using 11-cm-long, pH 5 to 8, precast immobilized pH gradient strips (IPG; Bio-Rad). The IEF parameters were successive steps of 300 V, 1 mA, and 5 W for 0.01 h; 300 V, 1mA, and 5 W for 4.5 h; 2,000 V, 1 mA, and 5 W for 5 h; and 2,000 V, 1 mA, and 5 W for 6.5 h. Following IEF, the IPG strips were equilibrated sequentially for 15 min each in 4 ml of equilibration buffer I (Bio-Rad) and buffer II (Bio-Rad). Second-dimension electrophoresis was performed on the strips in a Criterion cell (Bio-Rad) using 4 to 20% polyacrylamide gradient gels (Bio-Rad) for 1 h and 45 min at 115 V at room temperature in 25 mM Tris–250 mM glycine (pH 8.3)–0.1% sodium dodecyl sulfate (SDS). Gels were silver stained according to the protocol developed by Shevchenko et al. (41). In some cases, gels were stained with Sypro Ruby (Invitrogen, Carlsbad, CA).

Western blot analysis. *A. phagocytophilum* whole-cell lysates or Triton X-114-fractionated proteins were resolved by 1DE or 2DE and transferred to nitrocellulose at 15 V for 60 min using a Trans-Blot semidry transfer cell (Bio-Rad). Blots were screened using mouse monoclonal antibody (MAb) 20B4 (36, 40), 2H8, or 3G10 (kindly provided by J. Stephen Dumler of Johns Hopkins University) or rabbit polyclonal antiserum raised against rMSP2(P44) (21) followed by goat anti-mouse immunoglobulin G or goat anti-rabbit immunoglobulin G, respectively, conjugated to horseradish peroxidase (Cell Signaling, Boston, MA). The blots were incubated with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and exposed to film.

LC-MS/MS. Proteins of interest were excised from 2DE gels using the ProteomeWorks Plus Spot Cutter (Bio-Rad) and macerated. The gel pieces were vacuum dried and digested with trypsin in 25 mM NH_4HCO_3, pH 8, for 16 h at 37°C. Peptides were extracted by vortexing for 30 min in 5% CF_3COOH in 75% CH_3CN, followed by mixing with α-cyano-4-hydroxycinnamic acid in acetonitrile-water and analysis by liquid chromatography-electrospray ionization-ion trap tandem mass spectroscopy (LC-MS/MS) using an LC Packings capillary high-pressure liquid chromatography system (Dionex, Sunnyvale, CA) coupled with an ABI/MDS Sciex QSTAR XL quadruple time-of-flight mass spectrometer (AME Bioscience, Toroed, Norway) through a nanoelectrospray ionization source (Protona S. A., Geneva, Switzerland). Peptide fragment fingerprint data were used to search the NCBI-nr database using the MASCOT search program (Matrix Science). Protein assignments were made on multiple peptide hits. These analyses were performed in the University of Kentucky Center for Structural Biology.

Cloning and sequencing of *msp2*(*p44*) RT-PCR products. Total RNA was extracted from *A. phagocytophilum*-infected HL-60 cells and cDNA was synthesized as previously described (6). Primers 5′HV-F (5′-CGCTGCTCTTGGCAAAACC-3′) and 3′HV-R (5′-CCTTCAATAGTCTTAGCTAGTAACCC-3′ were used in conjunction with Platinum *Taq* High Fidelity polymerase (Invitrogen) to amplify the *msp2*(*p44*) HVR. Following an initial denaturing step at 94°C for 2 min, thermal cycling conditions were 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 68°C for 1 min, followed by a final extension at 68°C for 7 min. Amplicons were TA cloned into the pCR-4.0 TOPO vector (Invitrogen). Inserts were sequenced using vector-derived M13F and M13R primers at the Northwestern University Genomics Core Facility. Sequence analyses were performed using the Lasergene 7.1 software package (DNASTAR, Madison, WI).

Posttranslational modification analyses. In silico analysis of MSP2(P44)-18 for N-linked glycosylation motifs was performed using NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc/), while assessments for potential O-linked glycosylation sites were performed using NetOGlyc 3.1 (www.cbs.dtu.dk/services/NetOGlyc/) (24) and Support Vector Machines models (www.biosino.org/Oglyc) (25).

Cloning and expression of rMSP2(P44)-18. A 1319 bp DNA fragment encoding MSP2(P44)-18 of *A. phagocytophilum* HGE1 was amplified using Platinum *Taq* High Fidelity polymerase and primers p44-18-F (5′-GACGACGACAAGATGAGAAAAGGAAAGA-3′) and p44-18-R (5′-GAGGAGAAGCCCGGTAAAGCAAACC-3′); underlined nucleotides correspond to pET-51b(+) enterokinase (Ek)/ligation-independent cloning (LIC) vector-compatible sequences. After confirmation that it was of the expected size by agarose gel electrophoresis, the amplicon was purified using a Qiaquick PCR purification column (Qiagen, Valencia, CA). To generate compatible overhangs for subsequent cloning, 0.2 pmol of purified PCR product was treated with T4 DNA polymerase (Novagen, Madison, WI) at 22°C for 30 min, followed by heat inactivation of the enzyme at 75°C for 20 min. Next, 0.02 pmol of T4 DNA polymerase-treated PCR product was ligation-independently cloned into pET-51b(+) Ek/LIC-c (Novagen) per the

manufacturer's protocol. The recombinant plasmid was transformed into NovaBlue cells (Novagen). The plasmid was isolated from overnight cultures of a Novablue transformant using Qiagen's Qiaprep spin miniprep kit, and its sequence and junctions were verified. *Escherichia coli* BL21 (DE3) (Novagen) was transformed with the recombinant expression vector, and cultures of the transformant were grown in Luria-Bertani medium containing 50 mg ml^{-1} ampicillin at 37°C with shaking at 250 rpm. When the culture was in the mid-logarithmic phase of growth (optical density at 600 nm of 0.4), expression of rMSP2(P44)-18 was induced at 37°C for 3 h by adding IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM. Bacteria were harvested at 10,000 × *g* for 10 min at 4°C and resuspended in BugBuster (Novagen), and the soluble fraction and inclusion bodies were isolated according to the manufacturer's instructions. Protein concentrations were determined using the Bradford assay (Bio-Rad). Ten micrograms of uninduced and induced *E. coli* and the resulting inclusion bodies were resolved in 10% polyacrylamide gels and visualized by Coomassie blue staining. To confirm that the induced protein was the protein of interest, 10 μg of rMSP2(P44)-18 inclusion bodies and 10 μg of whole-cell lysates from uninfected and *A. phagocytophilum*-HGE1 infected HL-60 cells were screened via Western blotting using 20B4.

Monosaccharide analysis. Three hundred micrograms of rMSP2(P44)-18 was resolved in a 10% polyacrylamide gel. The rMSP2(P44)-18 band was excised and cut into multiple slices, which were dialyzed against water and freeze-dried overnight. Ten micrograms of inositol was added as an internal standard. Methyl glycosides were prepared by methanolysis in 1 M HCl in methanol at 80°C for 20 h. Next, all the methanolic HCl was taken into another tube (leaving behind the gels), dried, and re-N-acetylated with pyridine and acetic anhydride in methanol (for detection of amino sugars). The sample was per-O-trimethylsilylated by treatment with Tri-Sil (Pierce, Rockford, IL) at 80°C for 30 min, followed by hydrolysis with 1 M methanolic HCl for 16 h at 80°C to release sugars, which were subsequently derivatized with Tri-Sil. These procedures were carried out as described by Merkle and Poppe (32). Gas chromatography/MS analysis of the trimethylsilyl methyl glycosides was performed on an AT 6890N GC gas chromatograph interfaced to a 5975B MSD (Agilent Technologies, Foster City, CA), using a Supelco EC-1 fused-silica capillary column (30 m by 0.25 mm [inner diameter]; Supelco, St. Louis, MO). Monosaccharide analysis was performed at the University of Georgia Complex Carbohydrate Research Center, Athens.

RESULTS

Enrichment for *A. phagocytophilum* outer membrane proteins. We sought to assess the complement of *A. phagocytophilum* MSP2(P44) proteins expressed during cultivation in HL-60 cells and to determine whether MSP2(P44) is modified into multiple isoforms. To reduce the complexity of samples for MSP2(P44) proteomic analyses, we first enriched for *A. phagocytophilum* outer membrane proteins by temperature-dependent Triton X-114 phase partitioning (4, 39). This technique yields an upper aqueous phase containing hydrophilic proteins (hydrophilic liquid), a lower detergent phase enriched for membrane-anchored proteins and those carrying one to a few transmembrane domains (hydrophobic liquid), and an insoluble pellet consisting of more-complex membrane proteins (hydrophobic pellet). MSP2(P44) is predicted to carry 16 membrane-spanning domains, one of which is a C-terminal transmembrane anchor (19, 20). Western blot analysis using MSP2(P44)-targeting MAb 20B4 (36, 40) revealed the presence and absence of 42- to 44-kDa immunoreactive bands in the hydrophobic and hydrophilic liquid fractions, respectively (Fig. 1). 20B4 also recognized a series of 22- to 27-kDa bands in the whole-cell lysate and the hydrophobic liquid fraction. Because the hydrophobic pellet fraction could not be reconstituted in SDS sample buffer, it could not be resolved by 1DE.

***A. phagocytophilum* HGE1 expresses multiple isoforms of MSP2(P44)-18 during growth in HL-60 cells.** To assess whether MSP2(P44) is modified into multiple isoforms, the *A. phagocytophilum* hydrophobic pellet was resolved by 2DE.

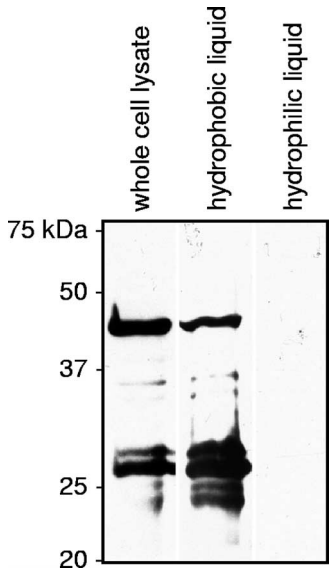
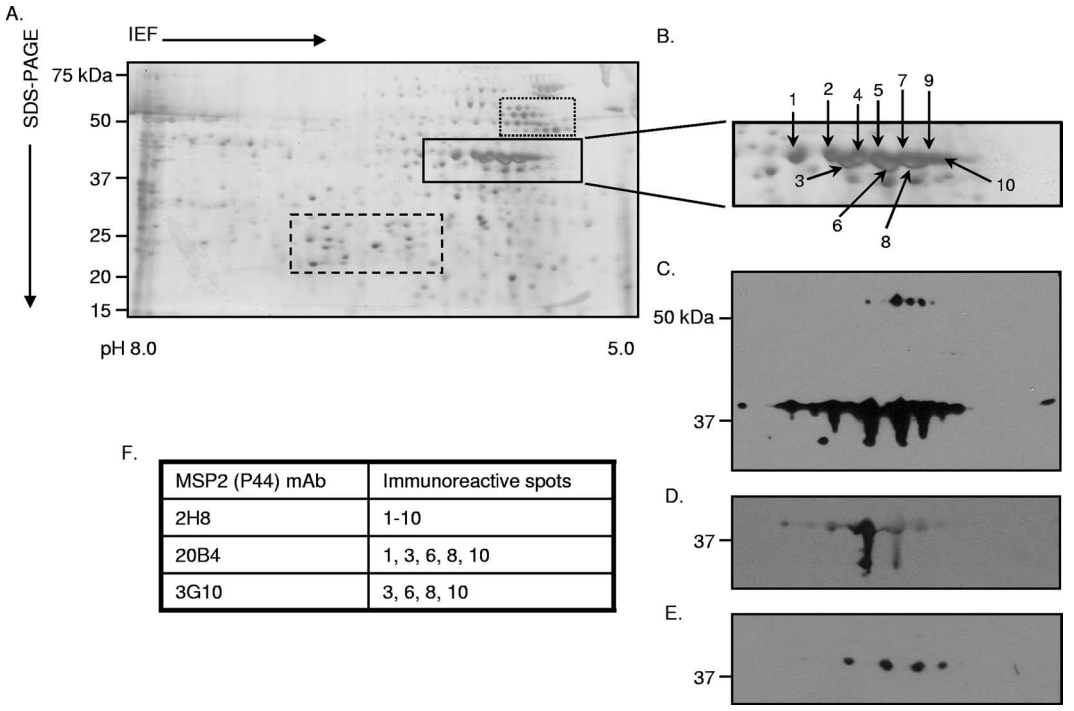


FIG. 1. MSP2(P44) MAb 20B4-screened Western blot of *A. phagocytophilum* HGE1 whole-cell lysate and Triton X-114-partitioned hydrophobic and hydrophilic liquid fractions. Ten micrograms each of *A. phagocytophilum* HGE1 whole-cell lysate and the hydrophobic liquid and hydrophilic liquid fractions obtained after Triton X-114 phase partitioning were screened via Western blot analysis using anti-MSP2 (P44) MAb 20B4.

Upon silver staining, a minimum of 10 spots having apparent molecular masses of 42 to 44 kDa and isoelectric points ranging from approximately 5.3 to 6.0 were detected in high abundance (Fig. 2A). To investigate whether these proteins corresponded to MSP2(P44), duplicate gels were analyzed by Western blotting using MAbs 2H8, 20B4, and 3G10, each of which had been raised against rMSP2(P44) (36, 40). 20B4 and 3G10 recognized several of the spots of interest, while 2H8 detected all proteins of interest plus a series of proteins varying in their isoelectric points that migrated with apparent molecular masses of 50 to 60 kDa (Fig. 2C to F). In addition to recognizing the 50- to 60-kDa and 42- to 44-kDa spots, rabbit polyclonal anti-rMSP2(P44) antiserum detected a series of 22- to 27-kDa spots (data not shown). 20B4 also recognized the 22- to 27-kDa proteins (data not shown). The multiple immunoreactive proteins therefore likely represented MSP2(P44) proteins. Because these were presumably derived from a single expression locus (1–3, 27, 28), we reasoned that they corresponded to a single MSP2(P44) protein that was posttranslationally modified into multiple isoforms. To test this hypothesis, 10 spots in the 42- to 44-kDa range were extracted and subjected to LC-MS/MS (Fig. 2B). A total of 29 different peptide masses were identified, many of which were recovered from multiple spots (Table 1). MSP2(P44) peptide masses were recovered from all 10 spots, thereby confirming their identities. All of the peptide masses matched to MSP2(P44)-18 (APH_1221 in the HZ genome) (18, 48), with 63.7% and 59.9% coverage of the mature protein and HVR, respectively (Table 1 and Fig. 3). Mature MSP2(P44)-18 is predicted to have a molecular mass of 43.1 kDa and an isoelectric point of 5.36, each of which is consistent with those of the selected spots of interest (Fig. 2A). The population of MSP2(P44)-18 trypsin



F.

MSP2 (P44) mAb	Immunoreactive spots
2H8	1-10
20B4	1, 3, 6, 8, 10
3G10	3, 6, 8, 10

FIG. 2. 2DE and Western blot analyses of *A. phagocytophilum* HGE1 MSP2(P44) proteins present in the hydrophobic pellet. Hydrophobic pellet fractions enriched for *A. phagocytophilum* outer membrane proteins were isoelectrically focused in IPG strips (pH 5.0 to 8.0), followed by resolution in the second dimension in gradient SDS-polyacrylamide (4 to 20%) gels. (A) Silver-stained gel. MSP2 proteins of ~42 to 44 kDa in size that were excised for LC-MS/MS identification or detected by anti-MSP2 antibodies are denoted by a solid box. A dotted box denotes a series of higher-molecular-mass proteins that are recognized by MAb 2H8. A dashed box denotes a series of proteins of ~22 to 27 kDa that were recognized by rabbit polyclonal anti-rMSP2(P44). (B) An enlarged view of the region of the silver-stained gel in panel A marked by a solid box. Numbered spots were excised and subjected to LC-MS/MS identification. (C to E) Spots in the predicted size range for full-length MSP2(P44) proteins were recognized by MAbs 2H8 (C), 20B4 (D), and 3G10 (E). (F) List of numbered spots denoted in panel B that were detected by each anti-MSP2(P44) MAb.

digestion-derived peptides was homogeneous with the exception of two pairs of peptides whose respective sequences each differed by a single amino acid (Table 1). The first pair was TSGKDIVQFAK and TSGKDFVQFAK (the differing amino acid is underlined in each peptide), which correspond to MSP2 (P44)-18 amino acids 171 to 181. The second pair was VAG DLTK and VAKDLTK, which represent amino acids 278 to 284. This slight variability in the MSP2(P44)-18 peptide profile is likely due to the fact that the *A. phagocytophilum* HGE1 population is nonclonal.

Because peptide masses recovered from the N- and C-terminal conserved domains would conceivably match all MSP2 (P44) paralogs and those derived from the HVR would be limited to a single or a select few paralogs, we focused on the HVR peptide masses to confirm whether each spot corresponded to only MSP2(P44)-18. Blast searches of the annotated *A. phagocytophilum* HZ genome and GenBank revealed that HVR peptide masses AVEISNSGIGKK, AVEISNSG IGK, YIVGAGDSSNAGTSLCGGKNQK, YIVGAGDSSNA GTSLCGGK, SSDTDTGVEK, and AQALHDFVSNTLSD GTK, which were collectively recovered from spots 1 to 6, 8, and 10, were 100% identical to only MSP2(P44)-18. The remaining HVR peptide masses corresponded to the C-terminal portion of the HVR and were 100% identical to not only MSP2 (P44)-18 but also several other paralogs. TIVAGLLAK, which occurs in the HVR immediately before the conserved C-terminal domain, is carried by 323 MSP2(P44) paralogs. Because this peptide mass was the only one to be recovered from spots 7 and 9, we cannot definitively state that spots 7 and 9 correspond exclusively to MSP2(P44)-18. Regardless, these data suggest that MSP2(P44)-18 is the predominant if not the only MSP2(P44) paralog expressed by *A. phagocytophilum* HGE1 during growth in HL-60 cells.

N-terminally truncated MSP2(P25)-18 isoforms are expressed by *A. phagocytophilum* HGE1 during growth in HL-60 cells. Because a series of 22- to 27-kDa immunoreactive proteins were detected using MAb 20B4 (Fig. 1) and because MSP2(P44)-18 is the predominant paralog expressed by *A. phagocytophilum* HGE1 during HL-60 infection, we next investigated whether such lower-molecular-mass proteins might represent truncated forms of MSP2(P44)-18. We used the hydrophobic liquid fraction because it contained enriched amounts of the proteins of interest (Fig. 4A). Upon 2DE, this fraction was observed to contain MSP2(P44)-18 isoforms, but overall it contained many fewer proteins than the hydrophobic pellet (Fig. 4A). A series of 20B4-immunoreactive 22- to 27-kDa proteins ranging in their isoelectric points from approximately 6.0 to 7.3 were present at greater concentrations in the hydrophobic liquid fraction than in the hydrophobic pellet (Fig. 4A and C). Seven of these spots were subjected to LC-MS/MS analysis (Fig. 4B). A total of 16 MSP2(P44)-18 peptide masses were identified, many of which were recovered from

TABLE 1. *A. phagocytophilum* HGE1 strain MSP2(P44)-18 peptide masses obtained from the hydrophobic pellet fraction

Region and peptide[a]	Spot no.[b]
N-terminal conserved	
IRDFSIR	1, 3, 6
AVYPYLKDGK	1, 3, 5, 8
AVYPYLK	2, 6
SVKLESHK	1
LESHKFDWNTPDPR	1, 6
FDWNTPDPR	3, 4, 5, 8
IGFKDNMLVAMEGSVGYGIGGAR	1, 3, 4, 6, 8
VELEIGYER	1, 2, 3, 4, 5, 6, 7, 8
GIRDSGSKEDEADTVYLLAK	3, 8
DSGSKEDEADTVYLLAK	1, 2, 4, 5, 6, 7
ELAYDVVTGQTDNLAAAKAK	1, 2, 3, 4, 6, 7, 8, 9
TSGKD**I**VQFAK	1, 3, 8
TSGKD**F**VQFAK	3, 6, 8, 9
D**F**VQFAK	4
Hypervariable	
AVEISNSGIGKK	1, 3, 4, 5, 6
AVEISNSGIGK	2, 8
YIVGAGDSSNAGTSLCGGKNQK	8
YIVGAGDSSNAGTSLCGGK	1, 2, 3, 4, 5, 6, 10
SSDTDTGVEK	1, 3, 8
AQALHDFVSNTLSDGTK	1, 2, 3, 4, 5, 6, 8
AVAGDLTK	1, 3, 5, 6, 8, 10
VA**K**DLTK	3, 5
KLTPEEK	1, 3, 4, 6
KLTPEEKTIVAGLLAK	8
TIVAGLLAK	1, 2, 3, 7, 8, 9
C-terminal conserved	
TIEGGEVVEIR	1, 2, 3, 4, 5, 6, 7, 8, 9
VVGDGVYDDLPAQR	1, 2, 3, 4, 5, 6, 7, 8
LVDDTSPAGR	3, 4, 5, 6, 8
TKDTAIANFSMAYVGGEFGVR	3, 4, 6

[a] Peptide sequences have been grouped into the N- or C-terminal conserved region or the central HVR of MSP2(P44). Individual amino acids that are different in otherwise identical peptides are denoted in bold.

[b] Spot numbers are consistent with those denoted in Fig. 2.

multiple spots (Table 2). Notably, only one peptide mass from the conserved N terminus was detected, which is in stark contrast to the 14 N-terminal peptide masses recovered from the hydrophobic pellet (Table 1). This peptide immediately precedes the HVR. With the exception of three new peptide masses (Table 2), all of the MSP2(P44)-18 peptide masses recovered from the hydrophilic liquid fraction matched to those recovered from the hydrophobic pellet. Seven MSP2 (P44)-18-specific peptide masses, six of which are identical to those recovered from the full-length isoforms plus DGDTTN RFAK, matched with 100% identity to MSP2(P44)-18. Thus, they collectively distinguish spots 2 to 7 as being derived from only MSP2(P44)-18. Spot 1 yielded only LVDDTSPAGR, which maps to the C-terminal conserved domain. Therefore, MSP2(P44)-18 cannot be distinguished as the only paralog from which this peptide mass could be derived. Overall, these results indicate that *A. phagocytophilum* HGE1 expresses a N-terminally truncated MSP2(P44)-18 isoform that is post-translationally modified into multiple species. Because these isoforms have apparent electrophoretic mobilities of 22 to 27 kDa and their derived peptide masses are identical to those of MSP2(P44)-18, we will refer to them as MSP2(P25)-18 isoforms.

Expression analyses of *msp2*(*p44*)*-18* during *A. phagocytophilum* HGE1 infection of HL-60 cells. To confirm that *msp2*(*p44*)*-18* is the predominant if not the only *msp2*(*p44*) paralog expressed by *A. phagocytophilum* HGE1 during infection of HL-60 cells, we used RT-PCR to amplify the *msp2*(*p44*) HVR and cloned and sequenced the amplicons. All 30 clones exhibited 100% nucleotide identity to each other (data not shown). The predicted protein encoded by the HGE1 *msp2*(*p44*)*-18* HVR consensus displays 99.3% identity to MSP2(P44)-18 (APH_1221) of the HZ strain (18, 48). The respective sequences differ only at amino acid 62, which is an isoleucine in MSP2(P44)-18 of *A. phagocytophilum* HZ and a phenylalanine in MSP2(P44)-18 of *A. phagocytophilum* HGE1. This difference has been noted by others, who observed distinctive switching between MSP2(P44)-18 isoforms of HGE2 that favored a phenylalanine at position 62 when passaged in HL-60 cells but favored isoleucine when passaged in the ISE6 tick embryonic cell line derived from *Ixodes scapularis* (3, 23). The fact that we recovered only *msp2*(*p44*)*-18* HVR RT-PCR clones supports our premise initially formulated from our LC-MS/MS data that MSP2(P44)-18 is the predominant if not the only paralog expressed by *A. phagocytophilum* HGE1 during cultivation in HL-60 cells. It also indicates a strong likelihood that the HVR peptide masses derived from hydrophobic pellet spots 7 and 9 and hydrophobic liquid spot 1 are derived from MSP2(P44)-18.

Posttranslational modification analyses of *A. phagocytophilum* HGE1 MSP2(P44)-18. The multiple MSP2(P44)-18 isoforms observed upon 2DE may result from posttranslational modifications, and such modifications likely contribute to their pathobiological functions. In silico analysis of MSP2(P44)-18 using NetNGlyc (www.cbs.dtu.dk/services/NetNGlyc/) revealed one possible N-linked glycosylation site at amino acid 415 (Asn-Xaa-Ser/Thr) (Fig. 3). NetOGlyc 3.1 (www.cbs.dtu.dk/services/NetOGlyc) (24) and Support Vector Machines models (www.biosino.org/Oglyc) (25) revealed one (amino acid 90) and three (amino acids 264, 265, and 268) potential O-linked glycosylation sites, respectively. Attempts to assess whether native MSP2(P44)-18 is glycosylated (13, 30, 31, 42, 43) were inconclusive (data not shown).

MRKGKIILGSVMMSMAIVMAGNDVRAHDDVSALETGGAGYFYVGLD
YSPAFSK*IRDFSIRESNGETKAVYPYLKDGKSVKLESHKFDWNTPD*
PRIGFKDNMLVAMEGSVGYGIGGARVELEIGYER*FKTK*GIRDSGSK
EDEADTVYLLAKELAYDVVTGQTDNLAAALAK*TSGKDIVQFAKAVE
ISNSGIGKK*VCETKRKDGDTTNRFAK*YIVGAGDSSNAGTSLCGGKN
***QKSSDTDTGVEKAQALHDFVSNTLSDGTK*NWPTSSETSKSNNDNAK**
AVAGDLTK*KLTPEEKTIVAGLLAKTIEGGEVVEIR*AVSSTSVMVNA
CYDLLSEGLGVVPYACVGLGGNFVGVVDGHITPKLAYRLKAGLSYQ
LSPEISAFAGGFYHR*VVGDGVYDDLPAQRLVDDTSPAGR*TKDTAIA
NFSMAYVGGEFGVRFAF

FIG. 3. MSP2(P44)-18 protein sequence. The full-length protein sequence of MSP2(P44)-18 is provided. Bold peptides were recovered from the hydrophobic pellet. Underlined peptides were recovered from the hydrophobic liquid fraction. The asparagine residue denoted by black shading and white text is predicted by NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc/) to be a potential N-linked glycosylation site. Amino acids shaded in gray correspond to those identified by NetOGlyc 3.1 (www.cbs.dtu.dk/services/NetOGlyc/) (24) and Support Vector Machines models (www.biosino.org/Oglyc) (25) as being potential O-linked glycosylation sites.

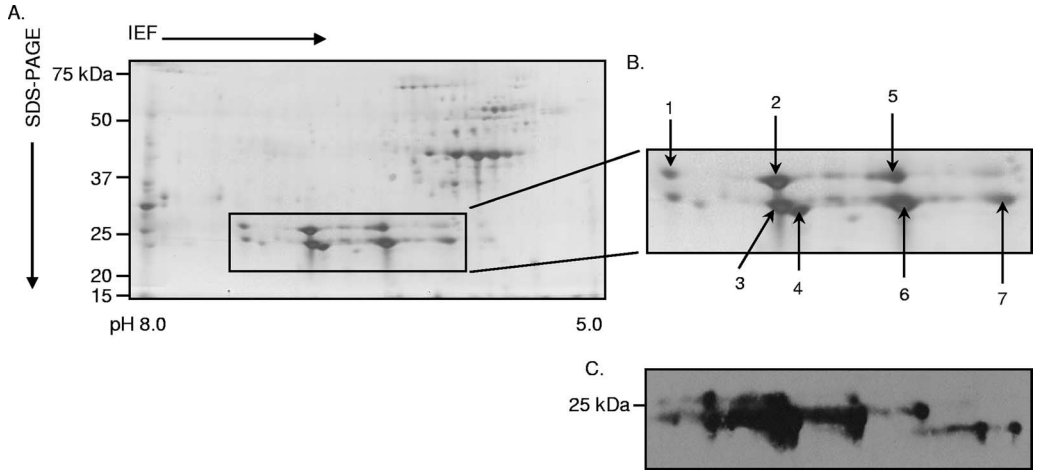


FIG. 4. 2DE and Western blot analyses of *A. phagocytophilum* MSP2(P25) proteins present in the hydrophobic liquid fraction. Hydrophobic liquid fractions enriched for *A. phagocytophilum* outer membrane proteins were isoelectrically focused in IPG strips (pH 5.0 to 8.0), followed by resolution in the second dimension in gradient SDS-polyacrylamide (4 to 20%) gels. (A) Silver-stained gel. MSP2 proteins of ~22 to 27 kDa in size that were excised for LC-MS/MS identification or detected by anti-MSP2 antibodies are denoted by a solid box. (B) An enlarged view of the region of the silver-stained gel in panel A marked by a solid box. Numbered spots were excised and subjected to LC-MS/MS identification. (C) Spots in the predicted size range for the N-terminally truncated MSP2(P25) proteins were recognized by MAb 20B4.

Carbohydrate composition analysis of rMSP2(P44)-18. While detection of carbohydrate modifications on native *Anaplasmataceae* proteins has proven difficult, gas chromatography has been successfully utilized to determine the sugar compositions of recombinant forms of *A. marginale* MSP1a, *E. chaffeensis* P120, and *E. canis* P140 (13, 31). We therefore cloned MSP2(P44)-18 and expressed it in recombinant form in *E. coli* (Fig. 5A). Proper expression of rMSP2(P44)-18 was confirmed by Western blot analysis using 20B4 (Fig. 5B). Monosaccharide composition analysis of rMSP2(P44)-18 determined that it is glycosylated, with glucose comprising the vast majority of the carbohydrate molar percentage, followed by considerably lesser amounts of galactose, xylose, and mannose and trace amounts of arabinose, fucose, and rhamnose (Table 3). The rMSP2(P44)-18 monosaccharide composition is highly similar to those of rMSP1a, rP120, and rP140 (13, 31).

TABLE 2. *A. phagocytophilum* HGE1 strain MSP2(P25)-18 peptide masses obtained from the hydrophobic liquid fraction

Region and peptide[a]	Spot no.[b]
N-terminal conserved	
TSGKDFVQFAK	2
Hypervariable	
AVEISNSGIGKK	2
AVEISNSGIGK	2, 5
DGDTTNRFAK	2, 6
YIVGAGDSSNAGTSLCGGKNQK	2, 6
YIVGAGDSSNAGTSLCGGK	2, 3, 4, 6
SSDTDTGVEK	3, 4, 5, 6, 7
AQALHDFVSNTLSDGTK	3, 6, 7
AVAGDLTK**K**	3
AVAGDLTK	2, 3, 6
AVAG**Y**LTK	3
KLTPEEK	2, 3, 4, 6, 7
TIVAGLLAK	2, 3, 4, 5, 6
C-terminal conserved	
TIEGGEVVEIR	2, 3, 4, 5, 6, 7
VVGDGVYDDLPAQR	2, 3, 4, 5, 6, 7
LVDDTSPAGR	1, 2, 3, 6, 7

[a] Peptide sequences have been grouped into the N- or C-terminal conserved region or the central HVR of MSP2(P25)-18. Peptide sequences not identified in MSP2(P44) spots from the hydrophobic pellet fraction presented in Table 1 are underlined. Individual amino acids that are different in otherwise identical peptides are denoted in bold.

[b] Spot numbers are consistent with those denoted in Fig. 4.

DISCUSSION

MSP2(P44)-18 is the dominant MSP2(P44) paralog expressed by *A. phagocytophilum* HGE1 during cultivation in HL-60 cells. At the time that this study was initiated, the HGE1 isolate had been passaged in HL-60 cells 92 times, yet the MSP2(P44) expression profile remained homogeneous for

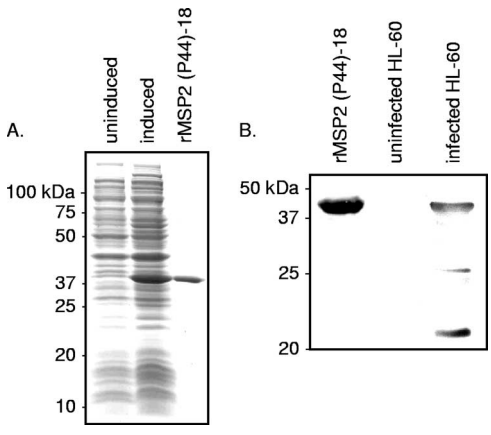


FIG. 5. Expression and Western blot analysis of rMSP2(P44)-18. (A) Coomassie blue-stained gel of whole-cell lysates of uninduced and IPTG-induced *E. coli* transformed to express rMSP2(P44)-18 and purified rMSP2(P44)-18. (B) Western blot of rMSP2(P44)-18 and uninfected and *A. phagocytophilum* HGE1-infected HL-60 cells screened with anti-MSP2(P44) MAb 20B4.

TABLE 3. Carbohydrate composition analysis of *A. phagocytophilum* HGE1 rMSP2(P44)-18

Glycosyl residue	mol%
Glucose	90.0
Galactose	4.3
Xylose	3.1
Mannose	1.3
Arabinose	0.6
Rhamnose	0.4
Fucose	0.2

MSP2(P44)-18. This finding is in agreement with previous observations that no to very limited changes occur in the *msp2*(*p44*) transcriptional profile during in vitro cultivation in the absence of immune pressure (7, 40). Our results obtained using the HGE1 strain are consistent with those for HZ and HGE2 (3, 23, 26, 48). The HZ strain was isolated from a patient in New York (38), while the HGE1 and HGE2 strains were recovered from patients in California (12). The consistency in MSP2(P44) expression by *A. phagocytophilum* isolates from geographically diverse regions in HL-60 cells suggests that MSP2(P44)-18 may have a fitness advantage in this host cell type in vitro in the absence of immune pressure. Indeed, *msp2*(*p44*)*-18* expression by HGE2 organisms disappears when the bacteria are transferred from HL-60 cells to ISE6 cells but reappears when they are returned to HL-60 culture (3, 23). This also occurs in vivo, as *msp2*(*p44*)*-18* transcription is downregulated upon transmission from mammals to ticks (48). Moreover, *msp2*(*p44*)*-18* is the dominant *msp2*(*p44*) transcript expressed in mammals during the early stages of infection regardless of animal species (horse or mouse), inoculation route (intraperitoneal or intravenous), or organism source (HL-60 culture or tick). This has been demonstrated using heterogeneous and clonal *A. phagocytophilum* strain HZ populations (26, 48). During persistent infection of immunocompetent laboratory mice, horses, and lambs, however, *A. phagocytophilum* expresses multiple, even complex mosaic MSP2 variants (16, 26, 45, 46). Further support is evidenced by the fact that simple *A. marginale* MSP2 variants have fitness advantages upon transmission to immunologically naïve calves and complex MSP2 mosaics arise during persistent infection only under selective immune pressure (35).

Additional evidence for MSP2(P44)-18 as a paralog with a fitness advantage in mammalian myeloid cells is provided by the observation that following inoculation of an isogenic *A. phagocytophilum* population into a horse, the percentage of expressed *msp2*(*p44*) PCR clones that were *msp2*(*p44*)*-18* positive gradually declined from 100% on day 0 postinfection to 0% on day 22 (26). Loss of *msp2*(*p44*)*-18* expression coincided with the emergence of anti-MSP2(P44)-18 antibodies. Despite the armamentarium of 113 *msp2*(*p44*) paralogs that afford the potential for greater antigenic diversification, however, *msp2*(*p44*)*-18* was reselected as the dominant paralog by day 44, as 78% of the clones corresponded to *msp2*(*p44*)*-18*, though the reemerged paralog carried several point mutations. Collectively, these studies demonstrate a predication for multiple *A. phagocytophilum* strains to express MSP2(P44)-18 during infection of HL-60 cells and neutrophils. Host-specific expression of outer surface proteins by other *Anaplasmataceae* members has been demonstrated, as certain P28 and P30 paralogs are expressed by *E. chaffeensis* and *E. canis*, respectively, during cultivation in mammalian and tick cell lines (42, 43).

In addition to full-length MSP2(P44)-18, *A. phagocytophilum* also expresses a series of 22- to 27-kDa N-terminally truncated isoforms. This phenomenon is not specific to *A. phagocytophilum* HGE1, as we have observed similarly truncated isoforms expressed by the NCH-1 and NCH-1A strains (M. J. Troese, M. Sarkar, and J. A. Carlyon, unpublished results). MSP2 (P44)-18 membrane topology modeling suggests that the protein contains 16 transmembrane domains that span the outer membrane to present the HVR as a large loop and five to seven smaller loops on the *A. phagocytophilum* surface (19). Truncated MSP2(P25)-18 lacks the first six of the predicted transmembrane domains, which is likely why the 22- to 27-kDa isoforms partition into the hydrophobic liquid fraction. Predicted transmembrane domain 7, which immediately precedes the HVR (19), is retained in MSP2(P25)-18, and therefore HVR surface presentation by the 22- to 27-kDa isoforms is likely uncompromised.

MSP2(P44)-18 is modified into ≥10 different isoforms by glycosylation and possibly by other types of posttranslational modifications. It is presumably a glucan because rMSP2 (P44)-18 is primarily decorated by glucose when expressed in *E. coli*. This may be a common trait among *Anaplasmataceae* outer surface proteins, as identical observations have been made for rMSP1a, rP120, and rP140 (13, 31). The role of MSP2(P44) glycosylation is not known. However, because MSP2(P44) has been indirectly implicated as an adhesin and because chemical deglycosylation of rMSP1a significantly reduces its adhesive properties (13, 36, 44), it is tempting to speculate that MSP2(P44) is indeed an adhesin and that such function is attributable to it being glycosylated. While other *Anaplasmataceae* major surface proteins have been shown to be phosphorylated (42, 43), we were unable to prove this phenomenon for MSP2(P44)-18. Perhaps phosphate residues of MSP2(P44)-18 are inaccessible to enzymatic removal due to neighboring glycosyl modifications. Alternatively, MSP2 (P44)-18 may not be phosphorylated but instead may be modified by sulfate or another charged chemical group.

The biological significance of the apparent processing of MSP2(P44)-18 into MSP2(P25)-18 remains to be determined. Interestingly, MSP2(P44)-18 bears structural and sequence motif similarities to members of the autotransporter superfamily, which consists of more than 800 proteins that are ubiquitous among the *Proteobacteria* and are also found in *Chlamydia* spp. (8, 14, 17). Autotransporters are often multifunctional and play roles in adhesion, invasion, cell-to-cell spread, serum resistance, and proteoloysis. Although heterogeneous in sequence, autotransporters are structurally conserved and consist of a signal sequence, a passenger domain, and a carboxy-terminal translocation unit. Autotransporters cross the inner membrane by a *sec*-dependent mechanism to the periplasm. Next, the specific carboxy-terminal domain assembles into a β-barrel pore in the outer membrane through which the passenger domain is translocated to the bacterial surface. At the cell surface, the passenger domain and β domain can remain intact or can be cleaved into separate units. If cleaved, the passenger either remains noncovalently associated with the cell surface or is secreted. MSP2(P44)-18 has a signal sequence,

has the propensity to form a β-barrel pore (19), and appears to have a cleavable N terminus. Its extreme carboxy terminus carries a motif consisting of a terminal aromatic residue preceded at a periodicity of two by aliphatic or aromatic residues that is necessary for formation of the β-barrel translocation unit by all characterized autotransporters. The passenger domains of autotransporters carry the motif GG[A/L/V/I][I/L/V/Y]$_n$FXXN (where *n* is an arbitrary number of amino acids and X is any amino acid) (17). MSP2(P44)-18 carries a similar motif, as FXXN occurs at amino acids 86 to 89, 97 to 100, and 249 to 252, the first two of which are preceded by GGAG or GGAR. Notably, the adhesive capabilities of several autotransporters are linked to their being glycosylated (14).

Our investigation and others' in vitro and in vivo studies using the HZ and HGE2 strains (3, 23, 26, 48) collectively implicate MSP2(P44)-18 as a paralog that is heavily favored for *A. phagocytophilum* infection of mammalian myeloid cells in the absence of selective immune pressure. Ours is the first to reveal that *A. phagocytophilum* MSP2(P44) is posttranslationally modified into multiple isoforms and adds to a growing body of evidence that this is a shared phenomenon among the outer surface proteins of several *Anaplasmataceae* pathogens. It is also the first to demonstrate that a series of 22- to 27-kDa N-terminally truncated MSP2(P25)-18 proteins are produced during *A. phagocytophilum* cultivation in HL-60 cells. Though the pathobiological significances of glycosylation and N-terminal truncation of MSP2(P44) remain to be defined, these observed phenomena as well as predicted secondary structural aspects of and sequence motifs carried by MSP2(P44) hint that it may function as an adhesin and/or autotransporter.

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